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A new species of clawed frog (genus *Xenopus*) from the Itombwe Massif, Democratic Republic of the Congo: implications for DNA barcodes and biodiversity conservation.

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Abstract

Here we describe a new octoploid species of clawed frog from the Itombwe Massif of South Kivu Province, Democratic Republic of the Congo. This new species is the sister taxon of *Xenopus wittei*, but is substantially diverged in morphology, male vocalization, and mitochondrial and autosomal DNA. Analysis of mitochondrial "DNA barcodes" in polyploid clawed frogs demonstrates that they are variable between most species, but also reveals limitations of this type of information for distinguishing closely related species of differing ploidy level. The discovery of this new species highlights the importance of the Itombwe Massif for conservation of African biodiversity south of the Sahara.

Key words: allopolyploid evolution, Albertine Rift, whole genome duplication, advertisement calls, DNA barcode, 16S, RAG1, RAG2

Introduction

Clawed frogs (*Xenopus* and *Silurana*) are widely used as model organisms for laboratory research and have a remarkable diversity and evolutionary history in sub-Saharan Africa. These frogs are unusual among vertebrates in their high number of polyploid species, frequency of independent polyploidization events, and range of ploidy levels, including diploid, tetraploid, octoploid, and dodecaploid species (Evans 2007; Evans et al. 2005; Evans et al. 2004; Kobel et al. 1996). All or almost all instances of polyploidization in clawed frogs occurred through allopolyploidization – genome duplication associated with hybridization between species (reviewed in Evans 2008). Analysis of mitochondrial and nuclear DNA suggests the existence of ancestral species that do not have known extant descendants with the same ploidy, including a diploid with 20 chromosomes, two diploids with 18 chromosomes, and three tetraploids with 36 chromosomes (Evans 2007). In other words, the genomes of possibly extinct species persist, in combination with other genomes, in extant allopolyploids. That these predicted species may actually be extant provides motivation for further fieldwork and species characterization in this group.

Here we report a new species of clawed frog from the Itombwe Massif of the South Kivu Province, Democratic Republic of the Congo (Fig. 1). As a resource for future taxonomic work and a supplement for existing

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mitochondrial and nuclear data that is already available for all species of clawed frog (Evans 2007; Evans et al. 2004), we also provide a new mitochondrial DNA barcode database for all known species of *Xenopus*.

Materials and methods

Field collection. The motivation for this study was an extensive examination of specimens by RCT that were collected in the Albertine Rift region. This effort identified specimens whose morphology was distinct from the known species. These specimens are archived at the Royal Museum for Central Africa at Tervuren, the Museum of Natural Sciences at Brussels, and also collections by Raymond F. Laurent that are not yet archived. However, because the species identity of these formalin preserved specimens remains unclear, we have elected to include in this description only those individuals for which species identity is unambiguous based on our molecular, cytogenetic, and vocal analyses. As a result the full range of this new species remains uncharacterized and we base this species description on individuals from only one locality.

In April 2006 BJE joined an expedition to the Itombwe Massif, South Kivu Province, which was organized by the Wildlife Conservation Society of the Democratic Republic of the Congo. Collections of clawed frogs were made at the town of Miki, which is about 50 km west of the northern tip of Lake Tanganyika and from the nearest motorized vehicle-accessible road (Fig. 1).

Molecular data, phylogenetic analysis. We estimated the phylogenetic position of the new species using molecular data from 2981 base pairs (bp) of four mitochondrial genes and cloned paralogs from 4233 bp of two tightly linked autosomal genes. Mitochondrial DNA sequences from 12S and 16S rDNA, $tRNA^{Val}$, and the cytochrome c oxidase subunit I (*CO1*) were analyzed. These sequences include samples from *X. wittei* from different parts of its range including two locations in Uganda and one location in Rwanda and also all other known (described or not) species of clawed frog (Evans et al. 2004). The autosomal DNA are from cloned paralogs of the *RAG1* and *RAG2* genes. These molecular data were collected using previously published primers (Evans 2007; Evans et al. 2004; Hajibabaei et al. 2005) and from all known species of clawed frogs, including those undescribed. Autosomal DNA sequences were obtained by sequencing individual clones of amplified paralogs.

Mitochondrial sequences were analyzed using a partitioned Bayesian analysis with separate data partitions for stem and loop regions of the rDNA genes, and a separate partition for *CO1*. A doublet model was used for the stem regions and a general time reversible model with a proportion of invariant sites and a gamma distributed rate heterogeneity parameter (GTR+I+Γ) was used for the loop partition and for the *CO1* partition. The GTR+I+Γ model for each of these partitions was selected using MrModeltest (Nylander 2004). Secondary structure of rDNA was inferred from an existing model for *X. laevis* (Cannone et al. 2002). Model selection for the autosomal DNA analyses was based on Bayes factors (Evans 2007; Nylander et al. 2004). Phylogenetic analyses were performed using MrBayes version 3.1.2 (Huelsenbeck & Ronquist 2001). The analysis of autosomal DNA presented here was previously published (Evans 2007), and in that study the new species was referred to as "*X.* new octoploid". All molecular data have been deposited in Genbank (accession numbers EU566830-51, EU588990, EU594660, EU599019-34, and others cited in Evans 2007; Evans et al. 2005; Evans et al. 2004). Barcode sequences (sensu Hebert et al. 2003a; Hebert et al. 2003b) were obtained for all species of clawed frog, including geographic sampling for some, with reserved keyword BARCODE applied by Genbank to partial mitochondrial *CO1* sequence accessions for 16 species from 22 localities that have vouchers, including the holotype and a paratype of the new species. Outgroup sequences were obtained from *Scaphiopus couchii*, *Rhinophrynus dorsalis*, and an unidentified species of *Hymenochirus*. All specimen data and barcode sequences, including electropherogram trace files, are available in the '*Xenopus* Barcode Profiles' file (project code BJEXS) in the Completed Projects section of the Barcode of Life Data System (BOLD; Ratnasingham & Hebert 2007).

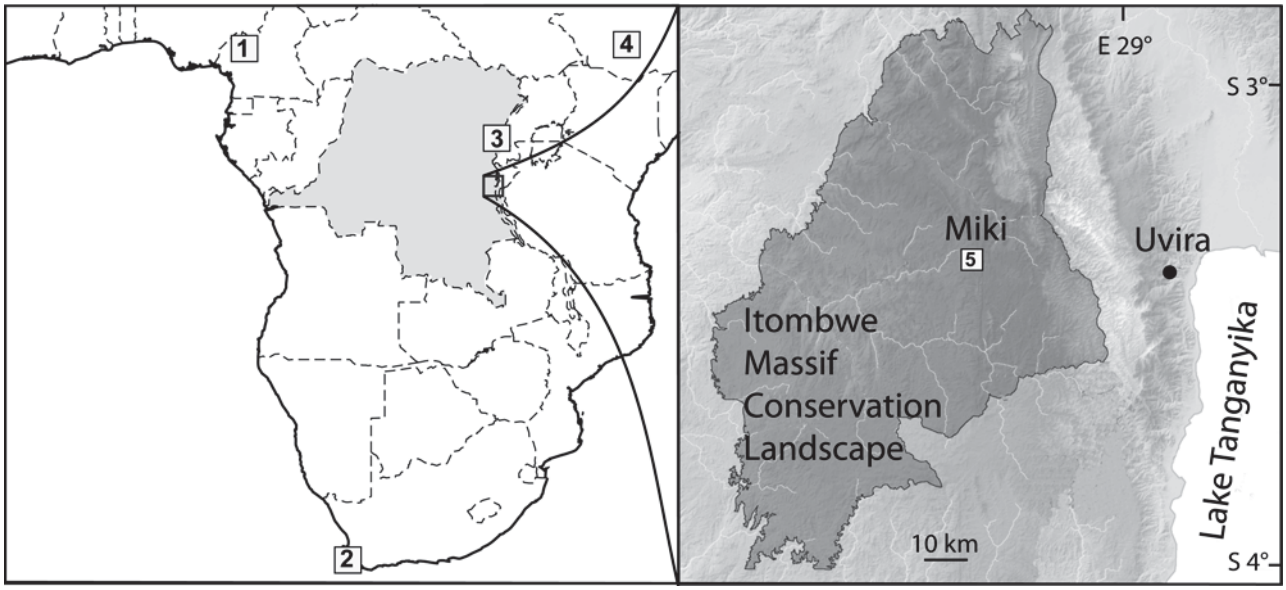

FIGURE 1. Distribution of selected *Xenopus* species with small geographic ranges. Numbered boxes indicate areas of interest. These include (1) the volcanic highlands of Cameroon (*X. longipes*, *X. amieti*), (2) lowland fynbos biome of South Africa (*X. gilli*), (3) the Albertine Rift highlands of the Eastern DRC, Uganda, Rwanda, and Burundi (*X. vestitus, X. wittei*, *X. ruwenzoriensis*), (4) the Bale Mountains of Ethiopia (*X. largeni*), and (5) the Itombwe Massif, South Kivu Province, Democratic Republic of the Congo (*X. itombwensis*). The right side indicates the location of the Itombwe Massif Conservation Landscape and the location of Miki, the type locality of *X. itombwensis*. (Image modified from the Wildlife Conservation Society.)

Analysis of morphology and male vocalization. Following Evans et al. (1998), seven external morphological measurements of the new species were taken as described in Table 1. In addition, we recorded the male advertisement call of the new species and also of the closely related species *X. wittei* and *X. vestitus*. Advertisement calling was evoked by priming males with human chorionic gonadotropin (Sigma; 100 international units on two consecutive days). Vocalizations were recorded approximately 5 hours after the second injection, at the start of the night cycle. An injected male and a sexually unreceptive female were placed in an 80 liter aquarium, two-thirds filled with dechlorinated water at 21°C. Recordings were made with a hydrophone (High Tech, Inc.; sensitivity= -164.5 dB re 1V/uPa), stored on CDs (Marantz, model CDR300), and analyzed using Signal software version 4.04.

Male clawed frogs produce species-specific advertisement calls composed of clicks; the opening of paired cartilaginous disks in the larynx accompanies each click (Yager 1992). Calls of some species are a single click whereas others produce trains of clicks called a trill; an uninterrupted succession of trills is termed a bout. As part of an ongoing study of phylogenetic variation in calls, we examined call variability and found that inter-individual variation within a species (from the same population) and the intra-individual variation at different times is minor compared to species-level differences in vocal parameters (data not shown). This observation enabled us to use the calls of one individual as representative of the species. Of course, this does not rule out the possibility of variation in vocalization among populations within a species. In this study, we recorded 12 calls from the new species, seven calls from *X. wittei*, and four calls from *X. vestitus*. Acoustic data were analyzed from approximately 200 clicks per species. In addition to measuring call duration and number of clicks per call, we also measured the inter-click interval (the time between two clicks in a trill), the sound frequencies with the two highest amplitudes (the dominant frequencies), the amplitude modulation (the fold difference in amplitude from the least intense click to the most intense click), and the bandwidth (the range of frequencies measured at 90% from the highest energy of the power spectrum).

Table 1. Morphological dimensions in millimeters of *X. itombwensis* including snout-vent length (SVL), the distance between the proximal edges of the eyes (IO), the distance between distal edges of the eyes (EO), the distance between the ends of the mouth (MO), the snout width at the anterior edge of the eyes (SW), the length from the anterior edge of the eyes to the end of the snout (SL), and the elbow to fingertip length (EF). When multiple individuals were measured, the mean, standard deviation (stdev) and maximum (max) of each measurement is listed.

Holotype (Male)	SVL	IO	EO	MO	SW	SL	EF
MCZ A-138192	31.8	3.3	8.3	9.2	7.4	2.6	13.9
Females (n=2)							
mean	35.7	3.5	8.8	8.8	7.0	2.6	13.3
stdev	3.0	0.5	0.1	0.6	0.5	0.0	0.9
max	37.9	3.5	9.1	8.8	7.5	2.8	14.1
Other Males (n=14)							
mean	30.2	3.3	7.9	8.2	6.7	2.2	12.6
stdev	3.3	0.4	0.6	0.8	0.8	0.3	1.5
max	37.4	4.2	9.0	9.6	8.2	2.9	15.3
Juveniles (n=2)							
mean	20.5	2.4	6.1	6.0	4.9	1.6	8.4
stdev	2.0	0.0	0.3	0.6	0.1	0.3	0.8
max	21.9	2.5	6.3	6.5	5.0	1.8	9.0

Cytogenetics. We developed a new method for karyotyping *Xenopus* using directly harvested liver tissue that does not require killing the animal or culturing cells. An animal was anesthetized by immersion in a 1% MS222 (Sigma) solution. The liver was accessed through a lateral incision and a small portion removed using cauterization. The incision was then closed with dissolvable surgical thread, and the frog was covered with a damp paper towel and placed on a floating weigh-dish to recover. Approximately 2.5 mm^3 of tissue was minced and treated with a 0.045M KCl hypotonic solution. After hypotonic treatment, large pieces of tissue were removed and the remaining cells were then spun down by centrifugation. The supernatant was removed, and then the cells were fixed by adding a 3:1 methanol:glacial acetic acid fixative solution dropwise while vortexing. The cells were then washed by spinning the sample down, removing the supernatent and resuspending it in fresh fixative. After two washes, resuspended cells were dropped onto ice cold pre-cleaned slides and then stained with a Giemsa based stain (8% volume/volume Giemsa to 6.86 pH phosphate buffer). Metaphase cells were viewed at 100x using brightfield microscopy (Zeiss Axioplan). Control karyotypes of *X. laevis* liver were also prepared to verify that metaphases containing the correct chromosome count (36) could be obtained with this method. The ploidy level of the new species was also verified by the number of divergent paralogs of *RAG1* and *RAG2* and the relationships among them.

Taxonomic account

***Xenopus itombwensis*, new species**
Itombwe Massif clawed frog
(Fig. 2)

Holotype: MCZ A-138192 (field number BJE 0275), adult male, Democratic Republic of the Congo, South Kivu Province, Miki Town, 3.35679º S, 023.69011ºE, approximately 2200 m above sea level. 21 April, 2006, Ben J. Evans.

Paratypes: Three adult males: MCZ A-138195 (BJE 0278), MCZ A-138196 (BJE 0283), MCZ A-138197 (BJE 0284), two juveniles, sex undetermined: MCZ A-138193 (BJE 0276), MCZ A-138194 (BJE 0277), same data as holotype.

Diagnosis: *Xenopus itombwensis* is a member of the *vestitus-wittei* subgroup of clawed frogs (Kobel et al. 1996) and can be distinguished from other members of this group by (1) unique but variable morphological coloration and smaller size (Table 1, Fig. 2), by (2) numerous temporal and spectral characteristics of the male advertisement call (Table 2, Fig. 3), and by (3) divergent mitochondrial and autosomal genes (Fig. 4). *Xenopus wittei* and *X. vestitus* are both medium sized clawed frogs, with female snout-vent length (SVL) typically 46 and 47 mm, respectively, and a maximum SVL of 61 and 55 mm, respectively (Kobel et al. 1996; Tinsley et al. 1979). Based on a small sample size of two females, *X. itombwensis* appears smaller, averaging approximately 35 mm (Table 1). Dorsal coloration of *X. wittei* is a uniform dark brown to chocolate with no spots and of *X. vestitus* is a marbeling of light silver-golden to bronze chromatophores over a brown background (Kobel et al. 1996). In contrast, some *X. itombwensis* individuals have a mottled pattern of brown spots that are slightly darker than the brown background (Fig. 2B,C).

The male advertisement call of *X. itombwensis* differs from the male advertisement calls of *X. wittei* and *X. vestitus* in that the call of the new species is much shorter (~600 milliseconds), and consists of two distinct components including a “fast trill” and a “slow trill” (Table 2, Fig. 3; Kobel et al. 1996; Vigny 1979). The dominant frequency of the fast trill component is similar to that of *X. wittei* but other acoustic characteristics of this part of the vocalization are different, and all parameters we measured from the slow trill component are distinct (Table 2). The most obvious difference between these vocalizations is the slow trill that follows vocalizations of the new species but not *X. wittei*. Other aspects of the slow trill are distinct from call parameters of *X. wittei* including the lower dominant frequencies, longer interclick interval, and lower number of clicks

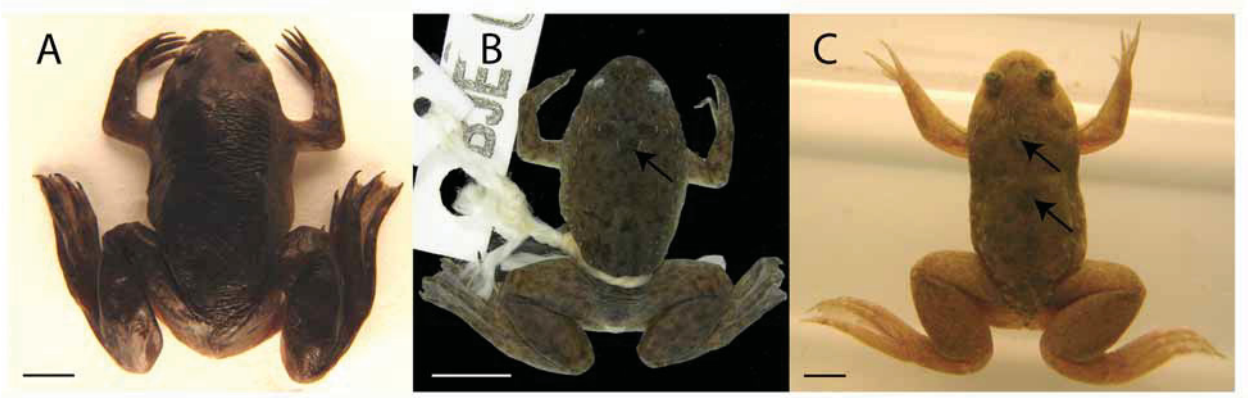

FIGURE 2. Type specimen and variation of *X. itombwensis.* (A) Holotype specimen MCZ A-138192 (field number BJE 0275), (B) Paratype MCZ A-138193 (field no. BJE 0276), and (C) a live unvouchered male individual. Arrows indicate dorsal spots that are not found in *X. wittei*. Scale bars are 5 mm. Photo credit for B: Jon Woodward.

(Table 2). The fast portion of the call of the new species is also unique in that the interclick interval is shorter than that of *X. wittei* and there are fewer clicks per call.

Evolutionary relationships to other clawed frogs; DNA barcodes. *X. itombwensis* and *X. wittei* are probably sister species derived from the same allo-octoploid ancestor. The paternal ancestor of *X. vestitus*, in contrast, shares recent common ancestry with the maternal ancestor of (*X. itombwensis* + *X. wittei*), but the maternal ancestor of *X. vestitus* is not as closely related to the paternal ancestor of (*X. itombwensis* + *X. wittei*). As a result, mitochondrial DNA of the *vestitus-wittei* group is not a clade (Fig. 4). On a molecular level, *X. itombwensis* is diverged from *X. wittei* and from *X. vestitus*. Because of the polyphyletic origin of this group, molecular divergence of mitochondrial DNA data (~3000bp) between *X. itombwensis* and *X. vestitus* is large – on the order of about 9% (uncorrected pairwise distance; hereafter *p*-distance). Mitochondrial divergence between *X. itombwensis* and *X. wittei* is about 4% *p*-distance. When only the "barcode" region of the mitochondria (*CO1*) is considered, interspecific divergence between *X. itombwensis* and *X. vestitus* and *X. itombwensis* and *X. wittei* is even higher – 17.2% and 8.3% *p*-distance, respectively. Paralogs of *RAG1* and of *RAG2* are also diverged among these species. Over both genes, the average divergence between the most closely related orthologous paralogs of *X. vestitus* and *X. itombwensis* is 1.6% *p*-distance and between orthologous paralogs of *X. wittei* and *X. itombwensis* is also 1.6% *p*-distance. These levels of divergence are typical for closely related species of clawed frog (Fig. 4; Evans 2007; Evans et al. 2005; Evans et al. 2004). The Genbank accession number of the barcode of the holotype is EU594660.

Description of the holotype: Holotype an adult male, small subocular tentacle present, comprising less than one third of the length of the eye. Claws present on toes I-III, prehallux prominent but without a claw. Size of new species slightly smaller than *X. wittei* and *X. vestitus* (Table 1; Kobel et al. 1996). Like *X. wittei* and *X. vestitus*, new species is octoploid with 8x=72 chromosomes (Fig. 5). Ventral surface of forelimbs and forearm with scattered black nuptial pads.

Color of the holotype in preservative: Dorsum homogeneous dark brown, transitioning laterally on flanks to a cream-colored venter; dorsal surface of head dark brown; dorsal surface of limbs dark brown; underside of head speckled with gray; venter cream colored with sparse small brown spots on ventral surface of hind limbs; ventral surface of hind feet cream-colored; holotype color in life unrecorded.

Variation and color in life: There are two dorsal color patterns evident in our sample of *X. itombwensis* from the type locality; the difference between these patterns is more subtle in preserved than in live specimens. The first pattern, which is present in the holotype, is a uniform brown to dark brown coloration (Fig. 2A) that is similar to *X. wittei*. The second pattern is a brown dorsal pattern with darker brown spots, sometimes with a dark dorsal band that is perpendicular to the body axis and situated caudal to the eyes but rostral

Table 2. Advertisement call characteristics of *X. vestitus, X. wittei,* and *X. itombwensis,* including call duration in milliseconds (CD), number of clicks per call (#C), amplitude modulation (AM), bandwidth (BW), interclick interval in milliseconds (ICI), and two dominant frequencies in kiloHertz (DF1 and DF2). See Methods for more information on these parameters. Measurements of the fast and slow trill portions of the *X. itombwensis* advertisement call are indicated separately.

X. vestitus	CD	#C	AM	BW	ICI	DF1	DF2
mean	1167.02	89.75	0.98	1035.53	12.72	2013.35	1733.42
stdev	220.17	16.92	0.01	1467.22	0.36	279.93	279.93
X. wittei							
mean	2036.03	67.83	0.76	1039.20	29.66	1381.71	1150.23
stdev	1286.35	42.10	0.12	245.10	3.14	107.59	292.28
X. itombwensis (fast trill)							
mean	334.64	20.13	0.61	446.42	16.65	1385.30	1432.86
stdev	44.96	3.44	0.30	268.30	1.68	213.61	190.83
X. itombwensis (slow trill)							
mean	318.75	8.79	0.65	450.57	35.67	894.64	970.56
stdev	67.93	1.04	0.12	249.52	8.06	202.40	229.74

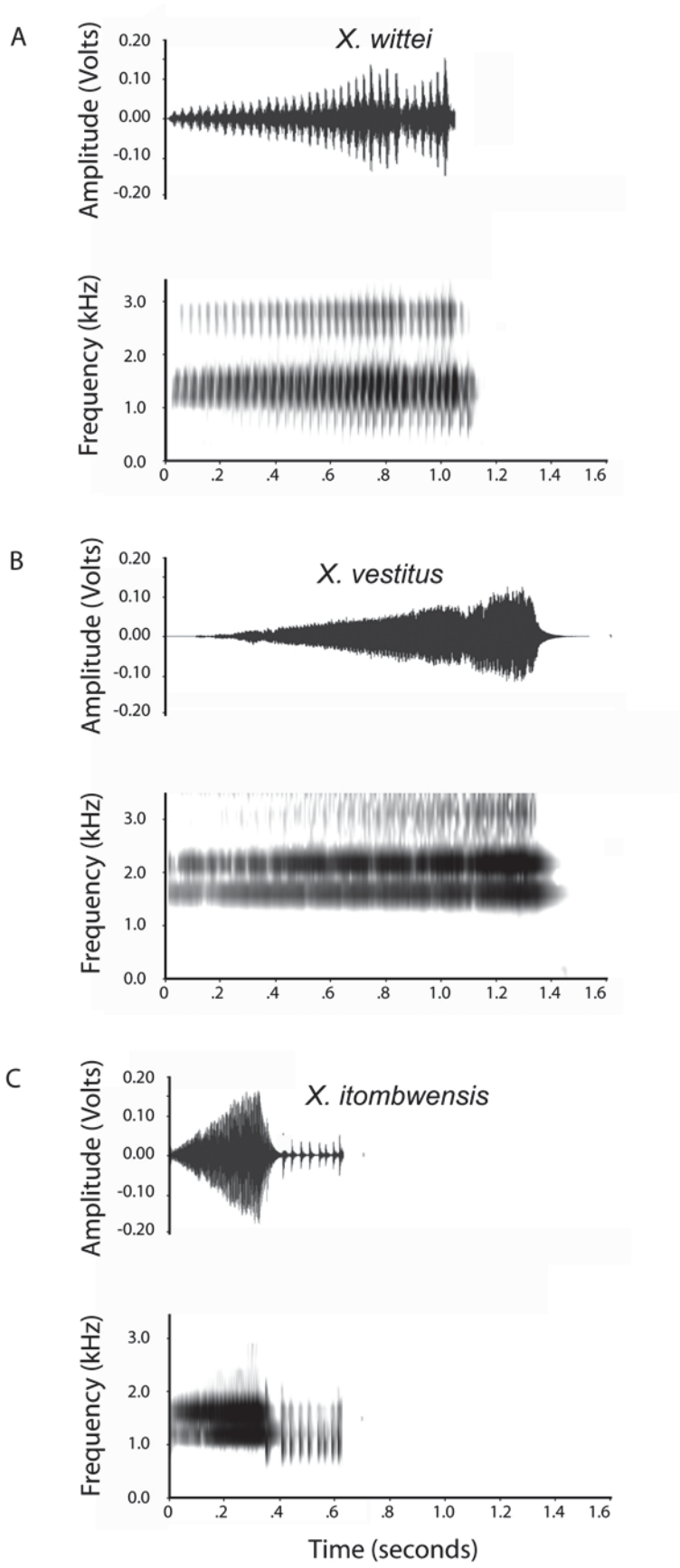

FIGURE 3. Male advertisement vocalization of (A) *X. wittei*, (B) *X. vestitus*, and (C) *X. itombwensis*. The slow trill portion of the *X. itombwensis* call (beginning at about 400 milliseconds) is a unique feature within the "vestitus-wittei" group.

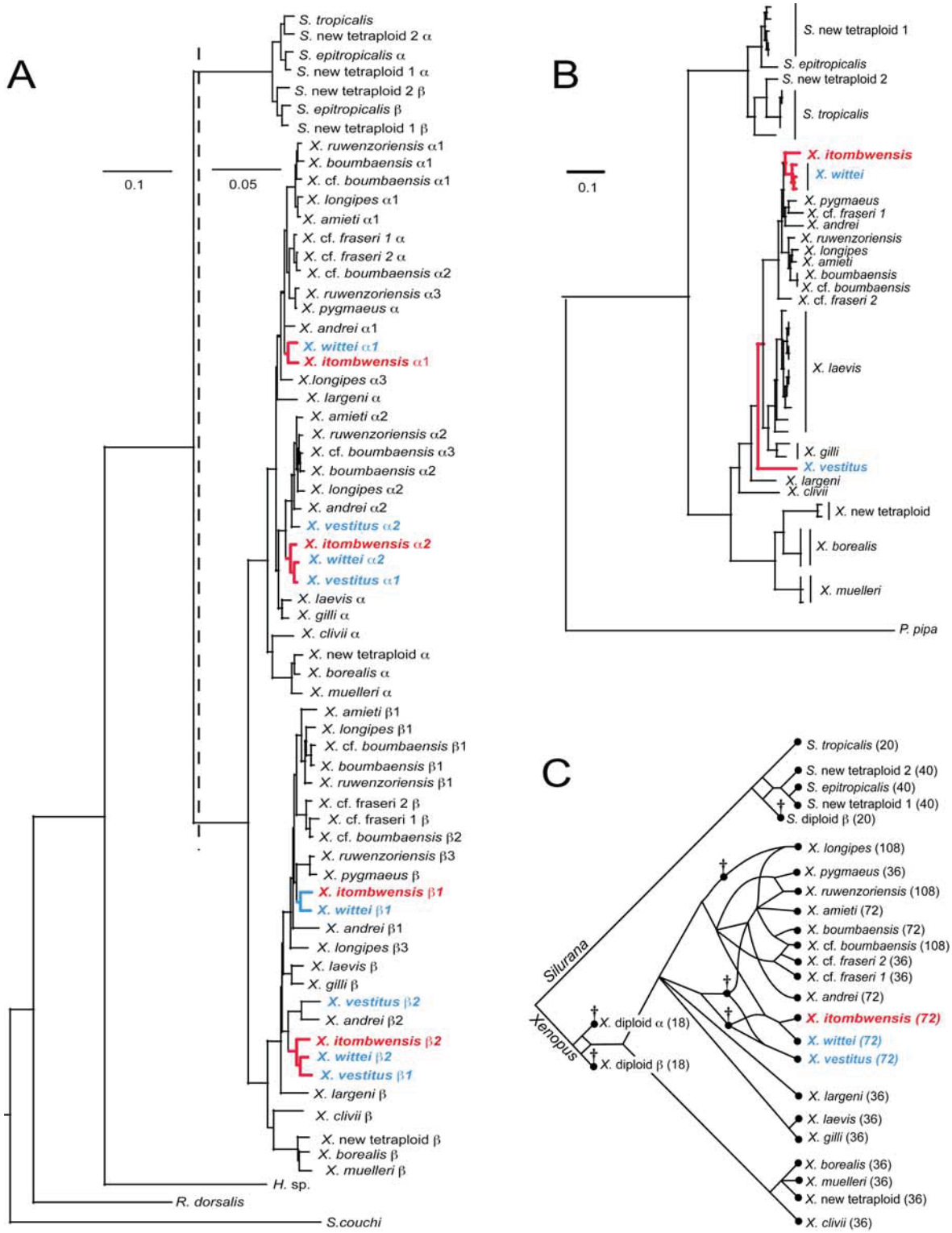

FIGURE 4. Evolutionary relationships of (A) combined data from two tightly linked nuclear loci (*RAG1* and *RAG2*) and (B) mitochondrial DNA illustrate a divergent but sister relationship of *X. itombwensis* and *X. wittei*. Nuclear loci but not mitochondrial loci illustrate a close relationship between (*X. itombwensis* + *X. wittei*) to half of the allopolyploid genome of *X. vestitus*. For clarity most posterior probabilities are omitted because they are similar or identical to those found elsewhere (Evans 2007; Evans et al. 2004). However, with reference to *X. itombwensis*, in (A and B) the red clades have over 95% posterior probability and the blue clade has over 80% posterior probability. (C) A species phylogeny illustrating bifurcating and reticulating evolutionary relationships in clawed frogs. The most recent common ancestor of *X. wittei* and *X. itombwensis* evolved through allopolyploidization of two tetraploid species. The number of chromosomes in each species is indicated in parentheses after each species name. (A) and (C) are modified from (Evans 2007).

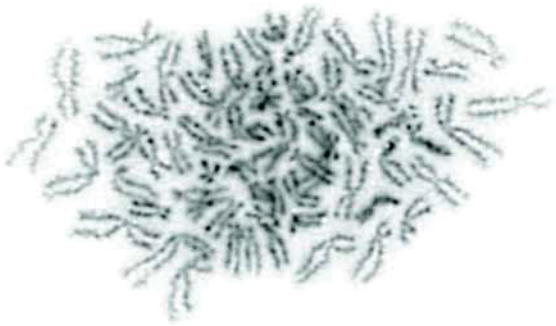

FIGURE 5. Karyotype of *X. itombwensis* illustrating octoploidy, 8x=72.

with respect to the forearms (Fig. 2B, C). The second color pattern is evident in three of the paratypes including one adult male: MCZ A-138194 (BJE 0277), and both of the juveniles: MCZ A-138193 (BJE 0276) and MCZ A-138195 (BJE 0278). In life, both the gular region and belly are cream colored and the leg and inguinal region are yellowish; sometimes there are small brown spots on the leg and inguinal region (Fig. 2C). Coloration at the margins of the lower jaw tends to be slightly darker than the venter, and varies from a dull gray under the head to a thin gray/brown line near the mouth. The barcode sequence of one of the paratypes (museum accession number MCZ A-138193, field number BJE 0276, barcode accession number EU566832) is the same as the holotype.

Size dimorphism: Females are larger than males; we suspect that the females we measured are not fully grown and that size dimorphism is greater than our measurements would suggest (Table 1).

Ecology and distribution: *Xenopus itombwensis* was collected only at the type locality and the extent of its distribution is unknown beyond this locality. These animals were locally abundant in standing water associated with mineral extraction in a region that was surrounded by mature forest and also mixed use agricultural areas.

Etymology: The new species is named after the plateau where it occurs – the Itombwe Massif of South Kivu Province, Democratic Republic of the Congo.

Discussion

Clawed frogs have been used on a global scale, earlier as a pregnancy assay (Shapiro & Zwarenstein 1934), and more recently as a model organism for biology (Cannatella & de Sá 1993; Dawid & Sargent 1988; Tinsley & Kobel 1996). Introduced populations of *X. laevis* are now established in Europe and the Americas (Fouquet & Measey 2006; Kuperman et al. 2004; Lobos & Jaksic 2005; Measey & Tinsley 1998; Tinsley & McCoid 1996). This group has an extraordinary evolutionary history that includes multiple independent instances of allopolyploidization, and a suite of species with restricted ranges in unusual sub-Saharan ecosystems. These evolutionary complexities make clawed frogs both a useful case study for exploring the effectiveness of biodiversity inventory approaches such as "DNA barcoding" and also an important group to consider for conservation purposes.

This study describes a new octoploid species of clawed frog from the Itombwe Massif, Democratic Republic of the Congo. Dorsal patterning – a polymorphic characteristic of the new species – is the most obvious morphological distinction between the new species and *X. wittei*, the dorsum of which is unpatterned. The new species is also slightly smaller than *X. wittei*, although this could in part be due to analysis of individuals

Table 3. Data for clawed frog species with and without "BARCODE" keyword in Genbank. Abbreviations include the Democratic Republic of the Congo (DRC) and National Park (NP).

	With "BARCODE" keyword			
Species	Field code	Museum ID	Accession number	Locality
S. epitropicalis	AMNH17275	MHNG 2644.56	EU566848	Kinshasa, DRC
S. new tetraploid 2	CAS207759	CAS207759	EU566851	Bioko Island, Equatorial Guinea
S. tropicalis	AMNH17271	MHNG 2644.55	EU566849	Freetown, Sierra Leone
S. tropicalis	L1720	ROM 19161	EU566850	Sapo National Park, Liberia
X. amieti	AMNH17267	MHNG 2644.54	EU566844	Galim, Cameroon
X. andrei	AMNH17255	MHNG 2644.51	EU566843	Longyi, Cameroon
X. borealis	AMNH17312	MHNG 2644.64	EU566842	Samburu, Kenya
X. boumbaensis	AMNH17284	MHNG 2644.57	EU566841	Maloundou, Cameroon
X. cf. *fraseri* 1	CAS207765	CAS207765	EU566845	Bioko Island, Equatorial Guinea
X. clivii	AMNH17252	MHNG 2644.50	EU566840	near Addis Ababa, Ethiopia
X. itombwensis	BJE00275	MCZ A-138192	EU594660	Miki, DRC
X. itombwensis	BJE00276	MCZ A-138193	EU566832	Miki, DRC
X. laevis	CAS168711	CAS168711	EU566839	Amani Pond, Tanzania
X. laevis	AMNH17259	MHNG 2644.52	EU566838	Jos, Nigeria
X. laevis	AMNH17263	MHNG 2644.53	EU566837	Lusaka, Zambia
X. laevis	AMNH17301	MHNG 2644.61	EU566836	near Blantyre, Malawi
X. laevis	AMNH17324	MHNG 2644.67	EU566846	Loubono, Congo Brazzaville
X. largeni	AMNH17292	MHNG 2644.59	EU566835	Kibre Mengist, Ethiopia
X. longipes	BJE00238	AMNH A168447	EU566834	lake Oku, Cameroon
X. muelleri	AMNH17308	MHNG 2644.63	EU566833	Ifakara, Tanzania
X. new tetraploid	AMNH17296	MHNG 2644.60	EU566847	Jos, Nigeria
X. wittei	CAS201664	CAS201664	EU566830	Bwindi Impenetrable NP, Uganda
X. wittei	AMNH17304	MHNG 2644.62	EU566831	Chelima Forest, Uganda
	Without "BARCODE" keyword			
Species	Field code	Museum ID	Accession number	Locality
S. new tetraploid 1	xen235	–	EU599019	Malemba, DRC
S. new tetraploid 1	xen236	–	EU599020	Makokou, Gabon
S. tropicalis	xen228	–	EU599021	Adiopo Doume, Cote d'Ivoire
S. tropicalis	xen231	–	EU599022	Uyere, Nigeria
X. borealis	xen226	–	EU599025	Kiambu, Kenya
X. cf. *boumbaensis*	AMNH17279	–	EU599023	Younde, Cameroon
X. cf. *fraseri* 2	Cr27	–	EU599024	Younde, Cameroon
X. gilli	Xs(2-3)	–	EU599026	Cape Province, South Africa
X. laevis	RT4	–	EU599029	Okavango Delta, Botswana
X. laevis	xen058	–	EU599030	Ngaoundere, Cameroon
X. laevis	xen232	–	EU599027	Shama, Rwanda
X. laevis	xen234	–	EU599028	Kitanga, Uganda
X. muelleri	xen225	–	EU599031	near Blantyre, Malawi
X. new tetraploid	MWK13208	–	EU588990	Guelta d'Archei, Chad
X. pygmaeus	AMNH17323	–	EU599032	Boende, DRC
X. ruwenzoriensis	AMNH17316	–	EU599033	Semiliki Valley, Uganda
X. vestitus	RT2	–	EU599034	Lake Mutanda, Uganda

from the new species that were not fully-grown. Molecular analysis of mitochondrial and nuclear DNA demonstrate that this species has a sister relationship to *X. wittei*, but that it is substantially diverged. The molecular divergence between the new species and all other *Xenopus* exceeds 8% at the *CO1* gene of the mitochondria for example. Moreover, *p*-distances underestimate the actual divergence because they are not corrected for multiple substitutions. Unlike *X. wittei*, the new species has two components of its vocalization consisting of a fast and a slow trill. The duration of the fast trill is less than half as long as the trill length in *X. wittei* and the time between clicks is longer than in *X. wittei*. The combined number of clicks per call of the new species, including the fast and the slow portion, is lower than that of *X. wittei*.

Mitochondrial DNA barcodes and speciation by polyploidization. Mitochondrial DNA barcodes of the new species distinguish it from all other species of *Xenopus* (Table 3). Although these data generally perform well in distinguishing species of clawed frogs, there is at least one example where mtDNA barcodes underes-

timate species diversity: an identical barcode is shared by the octoploid *X. boumbaensis* and the dodecaploid individual *X.* cf. *boumbaensis* (based on 585 bp of *CO1* gene). Additionally, 2335 bp of sequence from another part of the mitochondrial genome – the 12S and 16S ribosomal genes and the intervening $tRNA^{Val}$ gene – are essentially identical, differing by only one insertion/deletion polymorphism in the 12S gene. Thus, the 5' end of the 16S gene, which has also been suggested as a useful marker for amphibian species (Vences et al. 2005), was also unable to distinguish these species (although our 16S sequence is about 75 bp shorter than that analyzed by Vences et al.). Low divergence in mtDNA and nuclear DNA suggests a very recent origin of *X.* cf. *boumbaensis* by allopolyploidization between an ancestor of *X. boumbaensis* and *X. fraseri* (Evans 2007). This caveat to the effectiveness of mitochondrial DNA barcodes only impacts allopolyploid species that were formed so recently that mutations have not yet accumulated between the species, which is *not* the case for the new species described here. We do not yet know whether *X.* cf. *boumbaensis* is in fact a valid species or just an isolated instance of hybrid induced polyploidy (Evans 2007; Evans 2008).

Limitations of mitochondrial DNA for understanding diversification of allopolyploid species have been previously discussed (Evans et al. 2004). These limitations are relevant to the Barcode of Life initiative which aims to use DNA sequences from the mitochondrial *CO1* gene as a high throughput approach for species delimitation and recognition (Hebert et al. 2003a; Hebert et al. 2003b). For this reason, when genetic material is available for analysis, we suggest that future species descriptions of clawed frogs compare nuclear DNA sequences, such as *RAG1* or *RAG2* (Evans 2007) in addition to mitochondrial DNA sequences such as the barcode database for all clawed frogs provided here (Table 3) or other mitochondrial sequences that are available for all species of clawed frog (Evans et al. 2004).

Xenopus and biodiversity conservation. Three species of clawed frog, *X. amieti*, *X. gilli*, and *X. longipes*, are listed as near threatened, endangered, and critically endangered, respectively (Fig. 1; IUCN 2004). *Xenopus amieti* is endemic to the volcanic highlands of Cameroon and is considered near threatened due to habitat destruction in its limited range. *Xenopus gilli* is endemic to the lowland fynbos biome of Cape Province, South Africa, and is threatened by habitat destruction and possibly by competition with sympatric populations of *Xenopus laevis laevis*. Hybridization with *Xenopus laevis laevis* has not substantially compromised the genomic autonomy of this species, suggesting that it remains an evolutionarily distinct target for biodiversity conservation (Evans et al. 1997; Evans et al. 1998). *Xenopus longipes* is known only from Lake Oku in the volcanic highlands of Cameroon and is considered critically endangered as a result of this limited distribution and the possibility of fish introduction into this lake.

The Itombwe Massif is an area of exceptional conservation value that supports a unique component of the Albertine Rift biodiversity hotspot (Myers et al. 2000). The diversity and extent of habitat types associated with this plateau are among the most significant in Africa (Doumenge 1998). This is in part because this plateau lies at the intersection of three major phytogeographical regions including the lowland forests of the Congo basin, the montane forests of the Albertine Rift, and the grasslands of eastern and southern Africa (White 1983). High altitude portions of this plateau also may have harbored rainforest refugia during dry periods associated with Pleistocene glacial maxima (Nichol 1999; Plana 2004). In addition to *X. itombwensis*, other species of frog are endemic to this plateau (Laurent 1964; Schiøtz 1999). Nearly half of the total montane bird fauna of Africa is found on the Itombwe Massif, including multiple endangered and endemic bird species such as the Prigogine's Nightjar (*Caprimulgus prigoginei*), the Congo Bay Owl (*Phodilus prigoginei*), and Schouteden's Swift (*Schoutedanapus schoutedeni*) (Birdlife International 2003; Collar & Stuart 1988; Omari et al. 1999; Prigogine 1985). Recent surveys confirm that populations of chimpanzees and gorillas still persist on the Itombwe Massif, but that multiple local populations have disappeared in recent decades (Hart & Mubalama 2005; Omari et al. 1999; Schaller 1963). *Xenopus itombwensis*, therefore, not only constitutes a distinct component of clawed frog diversity but also is emblematic of a unique dimension of African biodiversity. Human activities such as mining, hunting, agriculture, logging, and overgrazing by livestock continue to take a major toll on local biodiversity on the Itombwe Massif (Omari et al. 1999). International support for

conservation efforts in the Itombwe Massif Conservation Landscape (Fig. 1), therefore, would make substantial progress towards protecting this important component of African biodiversity.

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